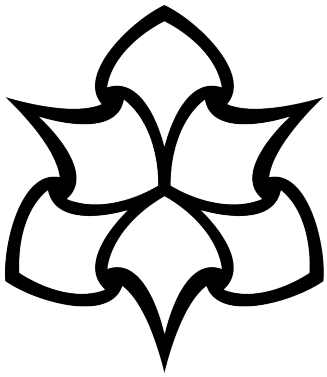

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The combined effects of water and nitrogen on the relationship between a native hemiparasite and its invasive host.

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Summary

- Stem hemiparasites are dependent on their hosts for water and nitrogen. Most studies, however, assess the influence of one factor on parasite:host associations, thus limiting our mechanistic understanding of their performance in nature.
- We investigated the combined effects of water and nitrogen (N) availability on both host (*Ulex europaeus*) and parasite (*Cassytha pubescens*).
- Parasite infection significantly decreased host shoot biomass and shoot:root ratio more severely in high water than low water, irrespective of N supply. Parasite stem [N] was significantly higher in high water than low water treatments, regardless of N supply, but parasite biomass didn't vary among treatments. Irrespective of water and N supply, infected plants had significantly lower total, root and nodule biomass, predawn and midday quantum yields, maximum electron transport rates, water potentials and nitrogen concentration [N]. Parasite $\delta^{13}C$ was significantly higher than that of the host.
- Our results suggest that stem hemiparasites can better extract resources from hosts when water availability is high, resulting in greater impact on the host in these conditions. Where hemiparasitic plants are being investigated as biocontrol for invasive weeds, they may be more effective in wetter habitats than in dry ones.

Key words: biomass, carbon isotope, chlorophyll fluorescence, hemiparasite, legume, nitrogen, *Ulex europaeus*, water potential.

Introduction

Parasitic plants comprise *c.* 1% of all angiosperms and occur on all continents apart from Antarctica (Heide-Jørgensen, 2013). They are an important group because of their direct and indirect impacts on ecosystems, communities and individual species across their global distribution (Press & Phoenix, 2005). For example, parasitic plants can increase nutrient cycling or affect plant community structure and diversity via their impacts at the host population level

(Bardgett *et al.*, 2006; Quested, 2008; Hartley *et al.*, 2015; Těšitel *et al.*, 2020). Numerous studies have investigated parasitic plant effects on their hosts (Press & Graves, 1995; Watling & Press, 2001; Press & Phoenix, 2005; Bell & Adams, 2011), but few have studied the impact of multiple environmental factors on these associations (e.g. Sui *et al.*, 2019). For instance, it is well known that abiotic factors such as nitrogen and water availability alter the impacts of parasitic plants on host performance (e.g. Cechin & Press, 1993; Le *et al.*, 2015), but they are rarely studied in combination, and where they have, they have largely focused on annual parasites of agricultural crops.

There have been several papers on the influence of nitrogen (N) on parasitic plant effects on their hosts, with mixed results. Some studies found that N had no influence on the association between *Striga hermonthica* and some cereal hosts (Gurney *et al.*, 1995; Aflakpui *et al.*, 1998, 2002, 2005; Sinebo & Drennan, 2001). By contrast, high N was found to reduce the negative effect of other annual root hemiparasites such as *Rhinanthus minor*, *Phtheirospermum japonicum* and *S. hermonthica* on hosts (Gibson & Watkinson, 1991; Cechin & Press, 1993, 1994; Irving *et al.*, 2019). High N was also found to reduce the negative effect of the annual stem holoparasite *Cuscuta* on its hosts (Jeschke & Hilpert, 1997; Shen *et al.*, 2013; but see Jeschke *et al.*, 1997). Cirocco *et al.* (2017) reported that in high N, the negative effect of the perennial stem hemiparasite, *Cassytha pubescens*, on root biomass of the native and invasive hosts, *Acacia paradoxa* and *Ulex europaeus*, was ameliorated and enhanced, respectively. In addition, this stem hemiparasitic vine strongly decreased nodulation of both these leguminous hosts, regardless of N supply (Cirocco *et al.*, 2017).

Surprisingly few papers have explored the influence of water on host:parasite associations, and most have investigated stem holo- or hemiparasitic vines. For instance, although the holoparasitic vine *Cuscuta australis* negatively affected photosynthesis of *Mikania micrantha*, irrespective of water supply, its negative impact on host stomatal conductance and transpiration was enhanced in low water conditions (Le *et al*., 2015). By contrast, Evans and Borowicz (2013, 2015) found that *Cuscuta gronovii* performed better and had a greater impact on host biomass in high water conditions. Similarly, the stem hemiparasite *Cassytha pubescens* also grew more vigorously and had a more severe impact on total biomass of the invasive host, *Ulex europaeus* in high water conditions (Cirocco *et al.*, 2016a). Cirocco *et al.* (2018) also found in a field study that the effect of *C. pubescens* on predawn quantum yield (F_v/F_m) of *U. europaeus* was strongest at the site with

highest water availability. Miller *et al.* (2003) investigated the impact of the mistletoe *Amyema miquelii* on the tree host *Eucalyptus largiflorens* at sites varying in soil salinity. Hosts at the sites with lowest salinity were more likely to be infected than those at high salinity sites. The impact of the mistletoe on host predawn water potential and $\delta^{13}C$, however, did not vary significantly across sites, or with parasite load (Miller *et al*., 2003).

Manipulating more than one biotic or abiotic factor is desired to better mimic complex field conditions. However, these studies are rare likely due to their large size and associated logistical difficulties and, to our knowledge, none have investigated stem hemiparasites (Matthies & Egli, 1999; Gao *et al.*, 2019; Jokien & Irving, 2019; Sui *et al.*, 2019). Two studies investigated the influence of water and nitrogen on host:parasite associations involving the annual root hemiparasite *Rhinanthus alectorolophus*, one with perennial hosts (Korell *et al*., 2019), and another with annual grasses (Těšitel *et al.*, 2015). Korell *et al.* (2019) found that water and nitrogen had no interactive effect on the association. In contrast, water and nitrogen did have an interactive influence on parasite effects on the annual grasses (Těšitel *et al.*, 2015). These contrasting results using the same parasite species may be related to differences in experimental design and/or host species. For example, annual hosts might be more sensitive to infection than perennial hosts because they have less capacity for resource storage to buffer parasite resource removal. With so few studies, however, it is difficult to generalise.

Here, we investigated the effect of water and nitrogen in combination on performance of a perennial, stem hemiparasite, *Cassytha pubescens,* and its impact on the perennial leguminous shrub, *Ulex europaeus*. We hypothesised that the parasite would negatively impact this major invasive shrub across water × nitrogen treatments, based on our previous field study which demonstrated a significant effect of the parasite regardless of environmental conditions (Cirocco *et al.*, 2018). We also hypothesised that the parasite would perform better and have a more pronounced effect on the host in high water treatments, regardless of N supply. This is because we previously found that high water availability enhanced the impact of the parasite on total biomass of *U. europaeus* (Cirocco *et al.*, 2016a), but nitrogen had no effect (Cirocco *et al*., 2017). To gauge parasite impacts on health of *U. europaeus*, we measured host photosynthetic performance, water and nutrient status, $\delta^{13}C$ and biomass. These same measures were quantified for *C. pubescens* to assess its performance on the host in the various treatments.

Materials and Methods

Study species

Ulex europaeus L. (Fabaceae) is a spiny shrub that can grow to 1–4 m tall and has a life span of 30 years (Hornoy *et al.*, 2011). Being a legume it can grow well on nutrient poor soils by obtaining nitrogen in reduced form via engagement with nitrogen-fixing bacteria, namely strains of *Bradyrhizobium* (Weir *et al.*, 2004; Rodríguez-Echeverría, 2010). It produces vast numbers of long-lived seed, has vigorous growth and can rapidly invade disturbed areas (Parsons & Cuthbertson, 2001). *Ulex europaeus* is native to the Iberian Peninsula, but over time has been introduced to all continents (apart from Antarctica), and has become so problematic that it is on the world's 100 worst invasive alien species list (Lowe *et al.*, 2000; see Hornoy *et al.*, 2013). *Cassytha pubescens* R. Br. (Lauraceae) is an Australian native, perennial, hemiparasitic vine (*c.* 0.5–1.5 mm in diameter) that has indeterminate growth and thus, can infect multiple hosts at any one time (Weber, 1981; Kokubugata *et al.*, 2012). It is an obligate parasite without roots that coils around and attaches to host stems not greater than *c.* 2.5 cm in diameter (McLuckie, 1924; Weber, 1981). *C. pubescens* is a generalist parasite and commonly infects *U. europaeus* in temperate southern Australia.

Experimental design

U. europaeus plants (*c*. 35 cm in height) were acquired from the field as described in Cirocco *et al.* (2017) in early September 2017. They were transplanted into 1.65-l pots containing sand with pH 4.75, which is similar to the pH of soil in the field where these parasite:host associations occur (Cirocco *et al.*, 2018). These plants were then randomly allocated into treatments (although treatments were not imposed until later; see below): high water or low water, and with high or low nitrogen availability. Each treatment combination had 10 uninfected and 10 *Cassytha* infected *U. europaeus*. Plants were also randomly allocated into 8 blocks with each block containing all factorial combinations.

U. europaeus were infected with the parasite in early November, 2017, according to Shen *et al.* (2010), and prior to water and nitrogen treatments being imposed. Briefly, host plants already infected with *C. pubescens* were positioned adjacent plants to be infected. The parasite was allowed to establish separate, independent connections with the experimental plants. Once

haustoria of the parasite were attached and appeared fully developed, the connection was severed from the donor plant. This process took 3 months (Supporting Information Fig. S1). During this time, plants were supplied monthly with 100 ml of liquid fertiliser (Nitrosol, Rural Research Ltd, Auckland, New Zealand; NPK 8 : 3 : 6), as per manufacturer's recommended dosage. Plants were grown in an evaporatively cooled glasshouse at The University of Adelaide.

All plants were subsequently transplanted into 5-l pots, containing the same soil medium, in late February 2018. Water × nitrogen treatments began at this time and plants were re-randomized within blocks fortnightly to negate any small light differences within the glasshouse (Supporting Information Fig. S1). Plants in the high and low water treatments were kept at 100% and 60% field capacity, respectively. Field capacity of the sand was determined using a modification of the filter-paper technique (Bouyoucos, 1929; see Cirocco *et al.*, 2016a for details). Plants in the high and low nitrogen treatments were supplied with standard or modified Hoagland's solution, respectively (refer to Cirocco *et al.*, 2017 for details). As *U. europaeus* is a legume low nitrogen plants were not supplied with an N source ($Ca(NO_3)_2.4H_2O$ and KNO_3 were substituted with $CaCl_2$ and KCL, respectively). All plants were supplied with 100 ml of the relevant Hoagland's solution weekly for the first 6 weeks, and then fortnightly following that. Treatments ran for *c*. 3 months and the experiment ended in late May-early June 2018. As the latter is the transition between autumn and winter in the southern hemisphere, plants were supplied with a supplemental light source for the final 2 weeks of the experiment when chlorophyll fluorescence and water potential measurements were made.

Chlorophyll fluorescence and water potential (Ψ)

Predawn quantum yield (F_v/F_m) of both host and parasite (n = 7–8) was measured 81 days after treatments were imposed (DAT) with a portable chlorophyll fluorometer (MINI-PAM, Walz, Effeltrich, Germany) equipped with a leaf-clip (2030–B, Walz, Effeltrich, Germany). The MINI-PAM and leaf clip were also used to generate rapid light response curves (RLCs) (see Cirocco *et al.*, 2017 for details). The RLC measurements for both host and parasite were made between 10:00 and 13:30 h over 2 days (83 and 84 DAT). Efficiency of PSII in light (Φ_{PSII}) of host and parasite (n = 7–8) was recorded at the sixth light step of the RLCs (PPFD = 981 ± 5 μmol m^{-2} s^{-1}, n = 94). Maximum electron transport rate (ETR_{max}) for both host and parasite (n = 7–8) was calculated using the RLCs by the Win-Control 3 software (ver 3.25, Walz).

Midday water potentials (Ψ) were determined on freshly cut shoots of *U. europaeus* and tendrils of *C. pubescens* using a Scholander-type pressure chamber with a digital gauge (PMS Instrument Company, Albany, OR). Midday water potential measurements were made between 12:00 and 14:45 h over 3 days (n = 7–8).

Biomass, $\delta^{13}C$ *and nitrogen concentration*

A destructive harvest was conducted using the six healthiest blocks (including parasite) at the end of the experiment (93–112 DAT). Above and below ground biomass as well as nodules of *U. europaeus* and parasite stems (n = 6) were oven-dried at 60°C for seven days. Carbon isotope composition ($\delta^{13}C$) and N concentration of *U. europaeus* spines and tendrils of *C. pubescens* were determined by mass spectrometry (GV Instruments, Manchester, UK) and elemental analysis (CUBE Elemental Analyser, Elementar Analysensysteme, Hanau, Germany) at Flinders Analytical, Adelaide.

Statistical analysis

Variances were homogeneous for both host and parasite data, unless otherwise stated. Three-way ANOVAs were used to analyse host parameters. If no three-way interactions (infection × water × nitrogen) were detected for a particular host parameter, then two-way interactions were considered, if these were also not detected then significant independent effects were treated as valid. For example a valid independent effect of infection would signify that the parasite had an effect on the host parameter regardless of the water or nitrogen conditions. Parasite data were analysed with two-way ANOVAs testing for interactions between water × nitrogen. Again, if no two-way interaction was detected for a particular parasite parameter, then independent effects of water or nitrogen were considered. All analyses were conducted with JMP software v.4.0.3 (SAS institute Inc., Cary, NC, USA) and $\alpha = 0.05$.

Results

Host and parasite growth

There were no significant treatment interactions for total biomass of *U. europaeus*, but it was independently affected by infection (Table 1; Fig. 1a). On average, infection suppressed total biomass by 71% (Fig. 1b). Water also independently affected this parameter: total biomass (gram

dry weight) of low water plants (53.3 ± 6.7) was 16% lower than that of high water plants (63.7 ± 7.8) (Table 1). There was an infection × water interaction for shoot biomass (Table 1; Fig. 1d), with infected plants being around 75% smaller than uninfected plants in high water compared with 68% smaller in low water (Fig. 1e). No treatment interactions were found for root biomass, but it was independently affected by infection which resulted in root biomass being 67% lower, on average across all treatments (Table 1; Fig. 1f,g).

Similar to shoot biomass, there was an infection × water interaction for shoot:root ratio of *U. europaeus* (Tables 1, 2). Shoot:root ratio (S:R) of high water infected plants was 15% lower than that of high water uninfected plants, whereas S:R of low water infected plants was not significantly different from that of low water uninfected plants (Table 2). There were no treatment interactions detected for nodule biomass or nodule biomass g^{-1} root biomass, but these parameters were independently affected by infection and nitrogen (Tables 1, 2). Infection suppressed nodule biomass by 39%, but increased nodule biomass g^{-1} root biomass by 47% (Table 2). Nodule biomass (including on a g^{-1} root biomass basis) of low nitrogen plants was on average 20% higher than that of high nitrogen plants (Table 2). Parasite biomass (including on a g^{-1} host total biomass basis) was unaffected by water or nitrogen treatments (Table 3; Fig. 2a,b).

Photosynthetic performance and water status of host and parasite

There were no treatment interactions detected for F_v/F_m, Φ_{PSII} or ETR_{max} of *U. europaeus*, but these parameters were significantly affected by infection (Table 4; Fig. 3a,c,e). Infection suppressed host F_v/F_m, Φ_{PSII} and ETR_{max} by 3, 26 and 34%, respectively (Fig. 3b,d,f). Treatments had no significant impact on F_v/F_m, Φ_{PSII} or ETR_{max} of *C. pubescens* (Table 3; Fig. 4a,b,c). There were also no interactions detected for midday Ψ of *U. europaeus*, however, there were significant independent effects of infection, water and nitrogen for this variable (Table 4; Fig. 5a). On average, midday Ψ of infected plants was 15% lower than uninfected plants (Fig. 5b). In addition, low water and low nitrogen plants both had an 11% more negative midday Ψ relative to high water and high nitrogen plants, respectively (Fig. 5c,d). Midday Ψ of *C. pubescens* was significantly lower (by 30%) than that of its host ($F_{1, 47} = 84.5$; $P = <0.0001$) (Fig. 5e). There was no interactive effect of water × nitrogen on midday Ψ of the parasite, but it was significantly affected by water supply (Table 3; Fig. 5f). As found for the host, midday Ψ of *C. pubescens* in the low water treatment was 10% more negative than the parasite growing in high water conditions (Fig. 5g).

Carbon isotope and nitrogen status of host and parasite

There were no treatment interactions detected for $\delta^{13}C$ of *U. europaeus*, however, infection, water and nitrogen all had independent effects on this host parameter (Table 4). The $\delta^{13}C$ of uninfected plants was significantly lower than that of infected plants (Table 2). Moreover, $\delta^{13}C$ of *U. europaeus* in either high water or high nitrogen was significantly lower relative to those in low water or low nitrogen conditions, respectively (Table 2). Also, $\delta^{13}C$ differed significantly between infected *U. europaeus* and *C. pubescens* ($F_{1, 35} = 123$; $P = <0.0001$). $\delta^{13}C$ of *C. pubescens* (–28.2 ± 0.17‰) was significantly higher than that of infected *U. europaeus* (–30.2 ± 0.17‰) ($n = 24$). There was no interaction between water and nitrogen on $\delta^{13}C$ of *C. pubescens*, however, similar to the host, both these factors independently affected $\delta^{13}C$ of the parasite (Table 3; Fig. 4d). $\delta^{13}C$ of *C. pubescens* in high water (–28.4 ± 0.17‰) was significantly lower than in low water conditions (–27.9 ± 0.29‰) ($n = 12$). Parasite $\delta^{13}C$ in high nitrogen (–28.6 ± 0.15‰) was significantly lower than that of *C. pubescens* in the low nitrogen treatment (–27.8 ± 0.25‰) ($n = 12$).

There were no treatment interactions found for foliar (i.e. spine) N concentration of *U. europaeus*, but it was independently affected by infection (Table 4; Fig. 6a). Foliar nitrogen concentration of infected plants was 10% lower than that of uninfected ones (Fig. 6b). No water × nitrogen interaction was detected for parasite stem N concentration, however, it was independently affected by water (Table 3; Fig. 6c). Nitrogen concentration of *C. pubescens* in low water was 18% lower compared with that in high water (Fig. 6d).

Discussion

Our hypothesis that water and nitrogen would not have an interactive influence on the impact of *C. pubescens* on *U. europaeus* was supported by the majority of the data. Irrespective of water and nitrogen treatments, infection with the native parasitic vine strongly decreased total biomass of the invasive shrub by 70%. Similarly, Korell *et al.* (2019) found that *Rhinanthus alectorolophus* negatively affected growth of three perennial hosts, regardless of water × nitrogen treatments. We also observed that infection had a greater impact on shoot biomass and shoot:root ratio of hosts in high water than low water, irrespective of N supply. In contrast, Těšitel *et al.* (2015) found that *R. alectorolophus* grew better and had a stronger impact on aboveground biomass of maize and wheat in low water conditions (when N supply was high). This disparity between findings may be related to *R. aclectorolophus* being able to maintain sufficient stomatal conductance and resource

removal under water deficits (Těšitel *et al.*, 2015). Conversely, *C. pubescens* appears to have lower stomatal conductance (parasite $\delta^{13}C$ higher in low water) and resource removal (less parasite N in low water, Fig. 6d) and thus, less impact in low water conditions. Relative to invasive hosts, some native hosts may tolerate infection due to their physiology (e.g. less profligate resource use and supply to the parasite) while others may display defence at the haustorial interface (Cameron & Seel, 2007; Facelli *et al.*, 2020). Thus, although native hemiparasites may still provide a potential means of controlling invasive weeds, their impact will be greater when water is not limited, and may also decline as drought becomes more likely in some regions as a consequence of climate change (Sala *et al.*, 2000; Těšitel *et al.*, 2017).

The impact of *C. pubescens* on growth of *U. europaeus* may be partly due to its effect on host photosynthesis (Fig. 3b,d,f). In the current study, host F_v/F_m was 3% lower for infected *U. europaeus*, irrespective of water or nitrogen treatment. In contrast, Těšitel *et al.* (2015) found that *R. alectorolophus* grew more and had a greater negative effect on F_v/F_m of maize when water was low and nitrogen was high. Thus, more parasite growth may translate to greater impact on this host parameter, and as *C. pubescens* did not grow better in any of the treatments this may explain why findings differed between studies. In two glasshouse experiments, *C. pubescens* also had a significant negative impact on F_v/F_m of *U. europaeus*, irrespective of water treatment or host size (Cirocco *et al.*, 2016a, 2020). In addition, despite environmental variation across field sites F_v/F_m of infected *U. europaeus* was significantly lower (by 5–10%) relative to uninfected plants (Cirocco *et al.*, 2018). F_v/F_m of another invasive host, *Cytisus scoparius,* was also significantly lower when infected with *C. pubescens* in a glasshouse experiment (Shen *et al.*, 2010), but not when measured in the field (Prider *et al.*, 2009). In both field and glasshouse conditions, the native parasite had no significant impact on F_v/F_m of *Leptospermum myrsinoides* likely due to this native host's ability (possibly through co-evolution) to maintain photoprotective capacity and xanthophyll engagement in response to infection (Prider *et al.*, 2009; Cirocco *et al.*, 2015). Whether invasive hosts such as *U. europaeus* have insufficient photoprotective capacity to cope with infection, increasing susceptibility to chronic photoinhibition requires investigation.

Ulex europaeus ETR_{max} was also significantly lower (by 34%) when infected with *C. pubescens*, regardless of water or nitrogen treatment. We found a similar impact of infection on ETR_{max} of this host (36%), irrespective of environmental variation among three field sites (Cirocco *et al.*, 2018). In three glasshouse experiments, ETR_{max} and midday ETR of infected *U. europaeus* were

also both significantly lower regardless of variations in host size, nitrogen or light (Cirocco *et al.*, 2016b, 2017, 2020). Shen *et al.* (2010) also found that ETR_{max} was significantly lower for *Cytisus scoparius* when infected with *C. pubescens*. By contrast, *C. pubescens* had no significant impact on ETR_{max} of *A. paradoxa* regardless of nitrogen (Cirocco *et al.*, 2017). Although midday ETR of infected *L. myrsinoides* was 36% lower when grown in high light, it was unaffected by *C. pubescens* in low light grown hosts (Cirocco *et al.*, 2016b). Shen *et al.* (2013) found that photosynthesis of the invasive *Mikania micrantha* significantly declined in response to infection with the stem holoparasite *Cuscuta campestris*, irrespective of nitrogen. Jokinen & Irving (2019) found that for the *Orobanche minor*:*Trifolium pratense* native association, photosynthesis of infected plants was significantly lower than uninfected plants, regardless of manipulations of light and nitrogen. Negative effects of parasitic plants on host photosynthesis are typically attributed to parasite acquisition of resources, resulting in lower host nitrogen (Cirocco *et al.*, 2018; Jokinen & Irving, 2019), stomatal conductance (Shen *et al.*, 2010; Cirocco *et al.*, 2017), or both (Shen *et al.*, 2013). Infected plants in our study did show signs of water and nitrogen depletion (Figs 5b, 6b) and possibly lower stomatal conductance as suggested by infected plants having significantly higher $\delta^{13}C$ than uninfected plants (Table 2).

In our study we found that *U. europaeus* foliar (spine) N concentration was 10% lower in infected plants, irrespective of water or nitrogen treatment. Similar impacts of infection on *U. europaeus* were observed both in the field and in earlier glasshouse studies (Cirocco *et al.*, 2016a, 2018). In contrast, we have also found that *C. pubescens* had no significant impact on foliar N concentration of *U. europaeus*, irrespective of host size, light or nitrogen availability (Cirocco *et al*., 2016b, 2017, 2020). There is no clear reason for this disparity among results, but the parasite can clearly adversely affect foliar N of this invasive host. In contrast, foliar N of native hosts, such as *A. paradoxa* and *L. myrsinoides,* does not seem to be impacted by *C. pubescens* (Cirocco *et al.*, 2016b, 2017). Irving *et al*. (2019) also found that for the *Phtheirospermum japonicum*:*Medicago sativa* native association, host N was unaffected by infection with this root hemiparasite, irrespective of nitrogen supply. On the other hand, Jokinen & Irving (2019) found that shoot N concentration of *Trifolium pratense* was significantly lower in plants infected with *O. minor*, regardless of light and nitrogen treatments. In our experiment, parasite removal of N may partly explain the lower foliar concentrations of this nutrient for infected *U. europaeus,* but it is not clear why this is not always observed for this association.

Based on previous studies (Cirocco *et al.*, 2016a, 2018), our hypothesis that parasite impact would be greatest in high water treatments was supported. Parasite impact on shoot biomass of *U. europaeus* was 7% greater in high water relative to low water conditions (Fig. 1e). Cirocco *et al.* (2016a) similarly found that the significant negative effect of *C. pubescens* on shoot and total biomass of *U. europaeus* was 25% stronger in high water relative to low water treatments. The difference in the magnitude of the high water effect between the two studies may be due to the low water treatments not being low enough in the current study, compared with low water treatments in Cirocco *et al.* (2016a). The reason why *C. pubescens* has a greater effect on *U. europaeus* in high water conditions may be due to it being more difficult for the parasite to remove host resources in low water conditions. The parasite did have a significantly lower water potential in low water relative to high water, presumably to assist in resource uptake (Fig. 5g). This lower Ψ may also have led to the parasite lowering its stomatal conductance over the long-term as inferred from parasite $\delta^{13}C$ in low water being significantly higher (by 0.5‰) compared with high water (Table 3). Cirocco *et al.* (2016a) also found $\delta^{13}C$ of *C. pubescens* in low water to be significantly higher (by 1.5‰) relative to high water conditions. Also, comparing $\delta^{13}C$ of *C. pubescens* across three field sites, this parameter was significantly higher at the driest site (Cirocco *et al.*, 2018). Nitrogen stress can also lead to decreased stomatal conductance (Chapin, 1991), and this may have occurred for the parasite with significantly higher $\delta^{13}C$ in low nitrogen compared with high nitrogen treatments (Table 3). Decreases in parasite stomatal conductance would help ameliorate its water status to some extent, but impede resource uptake from the host. The fact that low water treatments resulted in significantly lower N for the parasite but not for the host (Fig. 6d) implies that the parasite has difficulty in extracting N from the host in low water relative to high water. This explanation does not seems to be confounded by a diluting or concentrating N effect resulting from changes in parasite or host growth, as parasite biomass per gram host dry weight did not change among treatments (Fig. 2b). Thus, all of the above, in terms of compromised parasite water-status, stomatal conductance and N concentration in low water, help explain why its degree of impact on host growth was less severe in these conditions.

Comparing parasite and host sensitivity to water availability, $\delta^{13}C$ of *C. pubescens* was significantly higher (by 2‰) than its host, *U. europaeus,* and similar results have been previously reported by our lab for both glasshouse (Cirocco *et al.*, 2016a, 2020) and field studies (Cirocco *et al.*, 2018). Thus, it seems that *C. pubescens* is more conservative in its water-use than infected *U.*

europaeus. By contrast, mistletoes typically have lower $\delta^{13}C$ (less conservative in water-use) relative to their hosts particularly as temperature increases (Bannister & Strong, 2001; Scalon & Wright, 2015). This difference might be due to mistletoes having higher leaf tissue water capacitance than their hosts, thereby enabling higher stomatal conductances and lower water-use efficiences than the plants they infect (Glatzel, 1983; Davidson *et al.*, 1989). Whereas, *C. pubescens* being a thin vine is likely more vulnerable to dessciation and probably does not share this feature with mistletoes, although confirmation is required. Another possibility explaining higher parasite $\delta^{13}C$ relative to the host is that *C. pubescens* is achieving a substantial level of heterotrophy (Cernusak *et al.*, 2004), however, this also requires investigation.

Conclusion

Irrespective of water and nitrogen, the native stem hemiparasite *C. pubescens* had a large and significant impact on growth of *U. europaeus*, one of the world's worst invasive species. We also found that water and nitrogen status of the parasite were higher in well-watered conditions, suggesting that it was better able to extract resources under these conditions and may explain why its impact on host shoot biomas and S:R was greater in high water. Our data continue to support the potential use of some native hemiparasites as biocontrol for major invasive shrubby weeds (Těšitel *et al.,* 2020) and suggest that parasite impact is likely to be greater in higher rainfall areas regardless of soil nitrogen conditions. However, as some regions become drier and warmer in the future (Klausmeyer & Shaw, 2009), we may expect the impact of hemiparasitic plants on host performance to decline.

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Author contributions

RMC and JMF conceived and designed the experiment. RMC performed the experiment and analysed the data. RMC, JMF and JRW interpreted the analysis and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Photos of first and second phases of experiment.

Table S1 *F* and sum of squares for host growth parameters.

Table S2 *F* and sum of squares for all parasite parameters.

Table S3 *F* and sum of squares for host physiological measures.

Figure legends

Fig. 1 (a) Total, (c) shoot and (e) root biomasses of *Ulex europaeus* either uninfected (white bars) or infected with *Cassytha pubescens* (light grey bars) growing in high (H) or low (L) water (W) and nitrogen (N) conditions. (b) Independent effect of infection on total biomass. (d) Infection × water effect on shoot biomass and (f) independent effect of infection on root biomass. Data are means ± 1SE; different letters signify significant differences; (a, c, e) $n = 6$, (b, f) $n = 24$ and (d) $n = 12$.

Fig. 2 (a) Parasite biomass and (b) parasite biomass g^{-1} host total biomass of *Cassytha pubescens* when infecting *Ulex europaeus* growing in high (H) or low (L) water (W) and nitrogen (N) conditions. Data are means ± 1SE and $n = 6$.

Fig. 3 (a) Predawn and (c) midday quantum yield and (e) maximum electron transport rate of *Ulex europaeus* either uninfected (white bars) or infected with *Cassytha pubescens* (light grey bars)

growing in high (H) or low (L) water (W) and nitrogen (N) conditions. (b), (d) and (f) Independent effect of infection on all three host parameters. Data are means ± 1SE; different letters signify significant differences; (a, c, e) n = 7–8, (b) n = 30–32 and (d, f) n = 31–32.

Fig. 4 (a) Predawn and (b) midday quantum yield, (c) maximum electron transport rate and (d) carbon isotope composition of *Cassytha pubescens* when infecting *Ulex europaeus* growing in high (H) or low (L) water (W) and nitrogen (N) conditions. Data are means ± 1SE and (a, b, c) n = 7–8 and (d) n = 6.

Fig. 5 (a) Midday shoot water potential (Ψ) of *Ulex europaeus* either uninfected (white bars) or infected with *Cassytha pubescens* (light grey bars) growing in high (H) or low (L) water (W) and nitrogen (N) conditions. (b), (c) and (d) Independent effect of infection, water (dotted bars) and nitrogen (hatched bars) on host Ψ, respectively. (e) Ψ of *U. europaeus* infected with *C. pubescens* relative to that of the parasite (independent species effect: i.e. water and nitrogen plants pooled for each species). (f) Ψ of parasite growing in all treatment combinations. (g) Independent effect of water on parasite Ψ. Data are means ± 1SE; different letters signify significant differences; (a) n = 7–8, (b, c, d) n = 31–32, (e) n = 31, (f) n = 7–8 and (g) n = 15–16.

Fig. 6 (a) Foliar nitrogen concentration (N) of *Ulex europaeus* either uninfected (white bars) or infected with *Cassytha pubescens* (light grey bars) growing in high (H) or low (L) water (W) and nitrogen (N) conditions. (b) Independent effect of infection on host N. (c) N of parasite growing in all treatments. (d) Independent effect of water on parasite N. Data are means ± 1SE; different letters signify significant differences; (a, c) n = 6, (b) n = 24 and (d) n = 12.

Table 1 Three-way ANOVA results (*P*-values) for the effects of infection with *Cassytha pubescens* (I), water (W) and nitrogen supply (N) on total, shoot and root biomass, shoot:root ratio (S:R), nodule biomass (Nod) and nodule biomass g^{-1} root biomass (Nod g^{-1} root) of *Ulex europaeus*.

	Total	**Shoot**	**Root**	**S:R**	**Nod**	**Nod g^{-1} root**
I	**<0.0001**	**<0.0001**	**<0.0001**	**0.016**	**<0.0001**	**<0.0001**
W	**0.022**	**0.006**	0.349	0.127	0.154	0.577
I × W	0.126	**0.037**	0.569	**0.033**	0.335	0.541
N	0.417	0.316	0.930	0.151	**0.021**	**0.006**
I × N	0.955	0.927	0.746	0.190	0.510	0.996
W × N	0.936	0.911	0.922	0.843	0.507	0.721
I × W × N	0.859	0.740	0.746	0.292	0.407	0.585
Block	0.743	0.776	0.834	0.096	0.932	0.118

Significant effects are in bold; *F* and sum of square values are presented in Supporting Information Table S1 and root biomass and Nod g^{-1} root data log transformed to achieve homoscedasticity.

Table 2 Shoot:root ratio (S:R), nodule biomass (Nod; g dry weight), nodule biomass g^{-1} root biomass (Nod g^{-1} root) and carbon isotope composition ($\delta^{13}C$; ‰) of *Ulex europaeus* either uninfected (minus) or infected (plus) with *Cassytha pubescens* growing in high (HW) or low water (LW) conditions and supplied (HN) or not supplied (LN) with nitrogen.

Treatment	**S:R**	**Nod**	**Nod g^{-1} root**	**$\delta^{13}C$**
HW/HN–	3.63 ± 0.26	0.763 ± 0.076	0.036 ± 0.005	–31.4 ± 0.23
HW/HN+	2.79 ± 0.27	0.573 ± 0.089	0.074 ± 0.011	–30.8 ± 0.33
HW/LN–	3.44 ± 0.31	1.05 ± 0.10	0.049 ± 0.005	–31.2 ± 0.18
HW/LN+	2.50 ± 0.26	0.674 ± 0.085	0.091 ± 0.009	–29.9 ± 0.36
LW/HN–	2.76 ± 0.10	0.828 ± 0.080	0.038 ± 0.003	–31.3 ± 0.23

LW/HN+	3.15 ± 0.26	0.408 ± 0.058	0.066 ± 0.009	–30.1 ± 0.35
LW/LN–	2.89 ± 0.33	0.928 ± 0.125	0.045 ± 0.004	–30.1 ± 0.58
LW/LN+	2.39 ± 0.38	0.531 ± 0.050	0.081 ± 0.011	–29.9 ± 0.23
Infection × Water				
HW–	3.53 ± 0.19**a**	N/A	N/A	N/A
HW+	2.65 ± 0.19**b**	N/A	N/A	N/A
LW–	2.83 ± 0.16**ab**	N/A	N/A	N/A
LW+	2.77 ± 0.25**b**	N/A	N/A	N/A
Infection effect				
Uninfected	3.18 ± 0.15	0.893 ± 0.051**a**	0.042 ± 0.002**a**	–31.0 ± 0.19**a**
Infected	2.71 ± 0.15	0.547 ± 0.039**b**	0.079 ± 0.005**b**	–30.2 ± 0.17**b**
Water effect				
HW	3.09 ± 0.16	0.766 ± 0.055	0.062 ± 0.006	–30.8 ± 0.18**a**
LW	2.80 ± 0.15	0.674 ± 0.059	0.058 ± 0.005	–30.4 ± 0.21**b**
Nitrogen effect				
HN	3.08 ± 0.13	0.643 ± 0.050**a**	0.053 ± 0.005**a**	–30.9 ± 0.17**a**
LN	2.81 ± 0.17	0.797 ± 0.061**b**	0.067 ± 0.006**b**	–30.3 ± 0.20**b**

No infection × water × nitrogen interaction for all parameters; significant infection × water effect only for S:R; significant independent effects of either infection or nitrogen on Nod, Nod g^{-1} root and $\delta^{13}C$; significant independent effect of water on $\delta^{13}C$. Different letters in bold signify significant differences (vertically), data are means ± 1SE (N/A = data are not applicable); for each treatment: $n = 6$, infection × water effect: $n = 12$ and for all independent effects: $n = 24$.

Table 3 Two-way ANOVA results (*P*-values) for effects of water (W) and nitrogen (N) supply on parasite biomass, parasite biomass g^{-1} host total biomass, predawn and midday quantum yield (F_v/F_m and Φ_{PSII}), maximum electron transport rates (ETR_{max}), midday water potential (MD Ψ), carbon isotope composition ($\delta^{13}C$) and stem nitrogen concentration [N] of *Cassytha pubescens* infecting *Ulex europaeus*.

	Parasite biomass	**Parasite biomass g^{-1} host**	**F_v/F_m**	**Φ_{PSII}**	**ETR_{max}**	**MD Ψ**	**$\delta^{13}C$**	**[N]**

W	0.411	0.516	0.971	0.742	0.619	**0.019**	**0.050**	**0.027**
N	0.172	0.748	0.452	0.691	0.846	0.330	**0.002**	0.658
W × N	0.419	0.398	0.113	0.657	0.581	0.518	0.154	0.681
Block	0.386	0.989	**0.032**	0.235	0.298	**0.008**	**0.037**	0.553

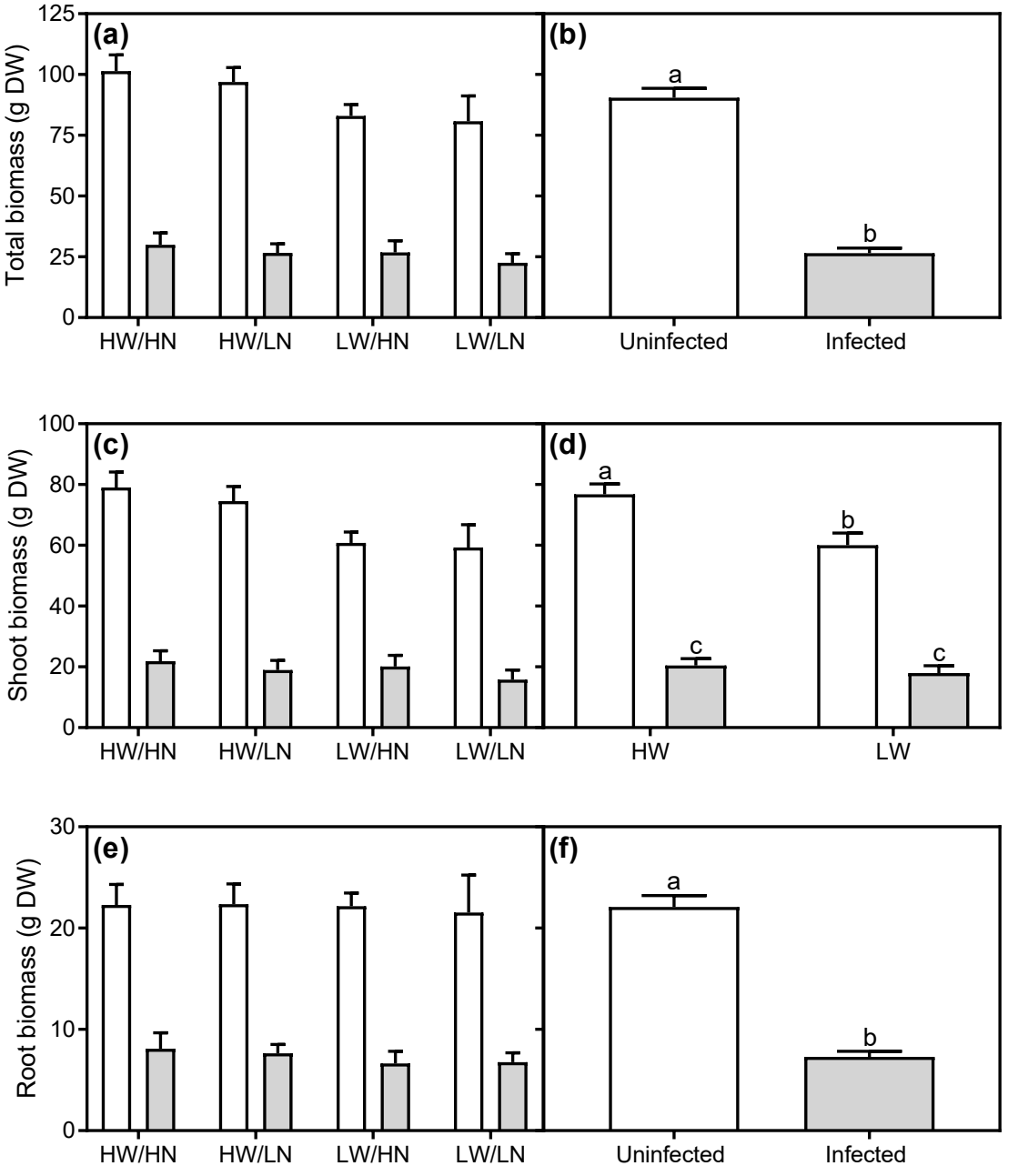
Significant effects are in bold; *F* and sum of square values are presented in Supporting Information Table S2.

Table 4 Three-way ANOVA results (*P*-values) for the effects of infection with *Cassytha pubescens* (I), water (W) and nitrogen supply (N) on predawn and midday quantum yield (F_v/F_m and Φ_{PSII}), maximum electron transport rates (ETR_{max}), midday water potential (MD Ψ), carbon isotope composition $\delta^{13}C$ and foliar nitrogen concentration [N] of *Ulex europaeus*.

	F_v/F_m	Φ_{PSII}	ETR_{max}	MD Ψ	$\delta^{13}C$	[N]
I	**<0.0001**	**<0.0001**	**<0.0001**	**0.0001**	**0.0009**	**0.002**
W	0.388	0.222	0.139	**0.003**	**0.046**	0.837

I × W	0.407	0.991	0.661	0.537	0.581	0.238
N	0.649	0.495	0.903	**0.003**	**0.011**	0.918
I × N	0.446	0.479	0.274	0.308	0.683	0.702
W × N	0.978	0.838	0.750	0.471	0.871	0.372
I × W × N	0.737	0.402	0.211	0.627	0.098	0.380
Block	0.537	0.361	0.332	0.203	0.135	0.599

Significant effects are in bold; *F* and sum of square values are presented in Supporting Information Table S3.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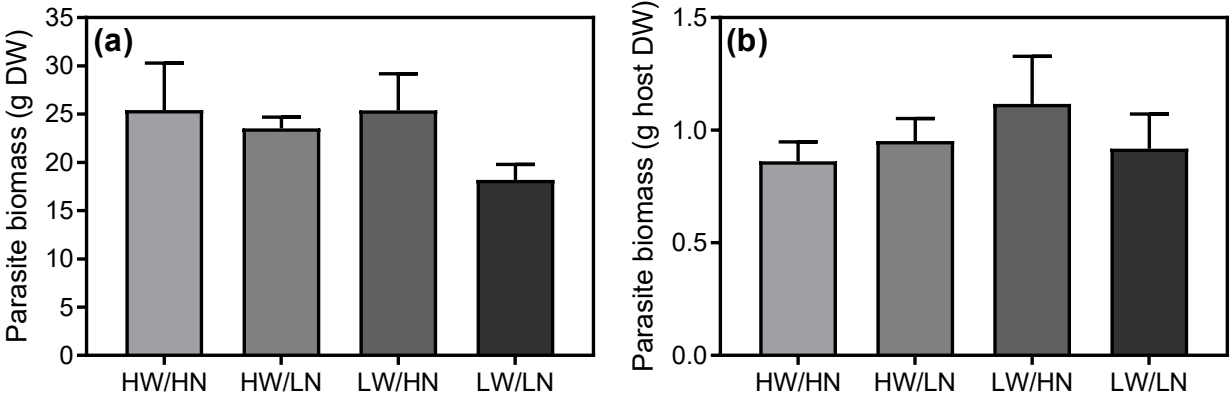
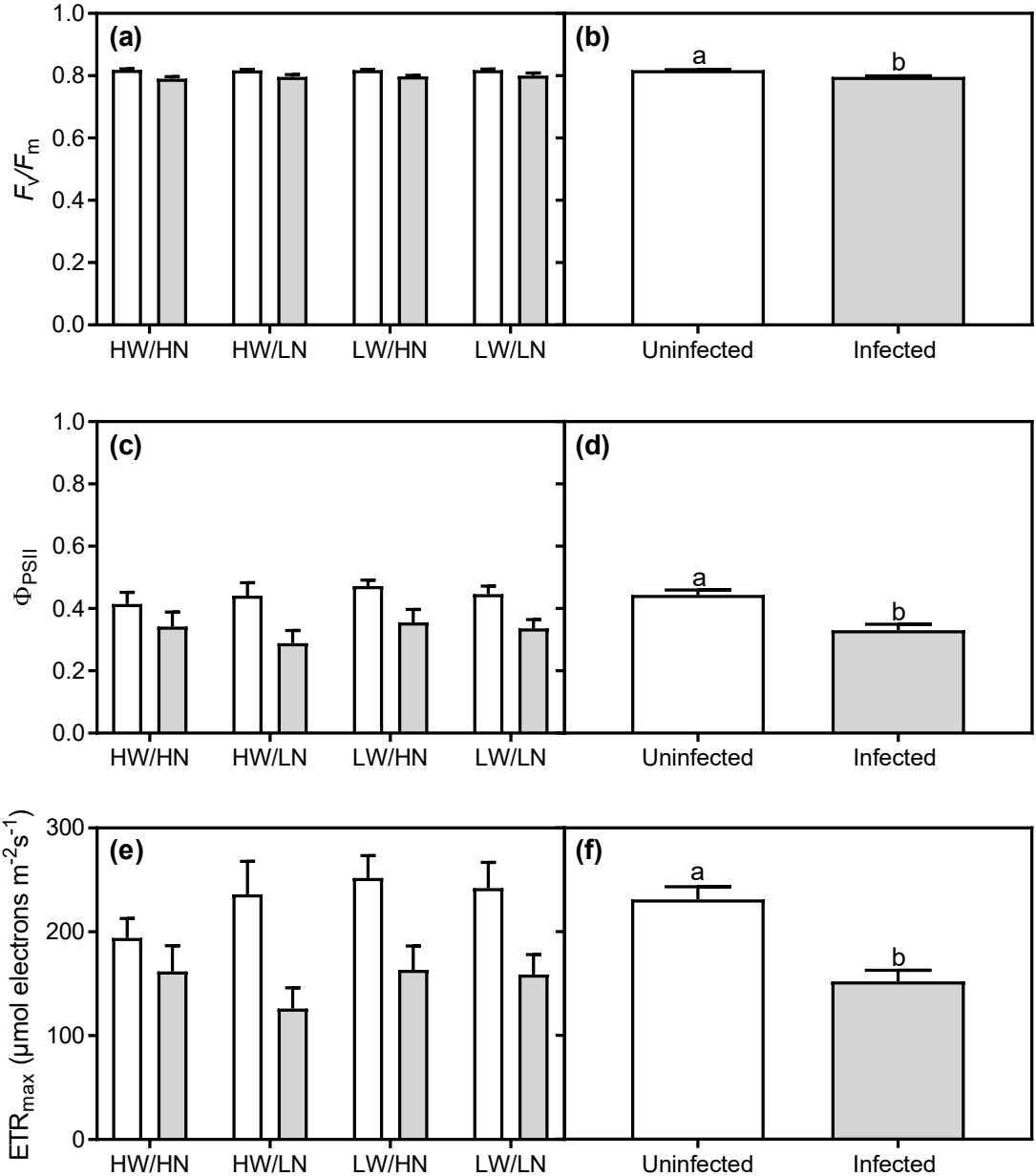
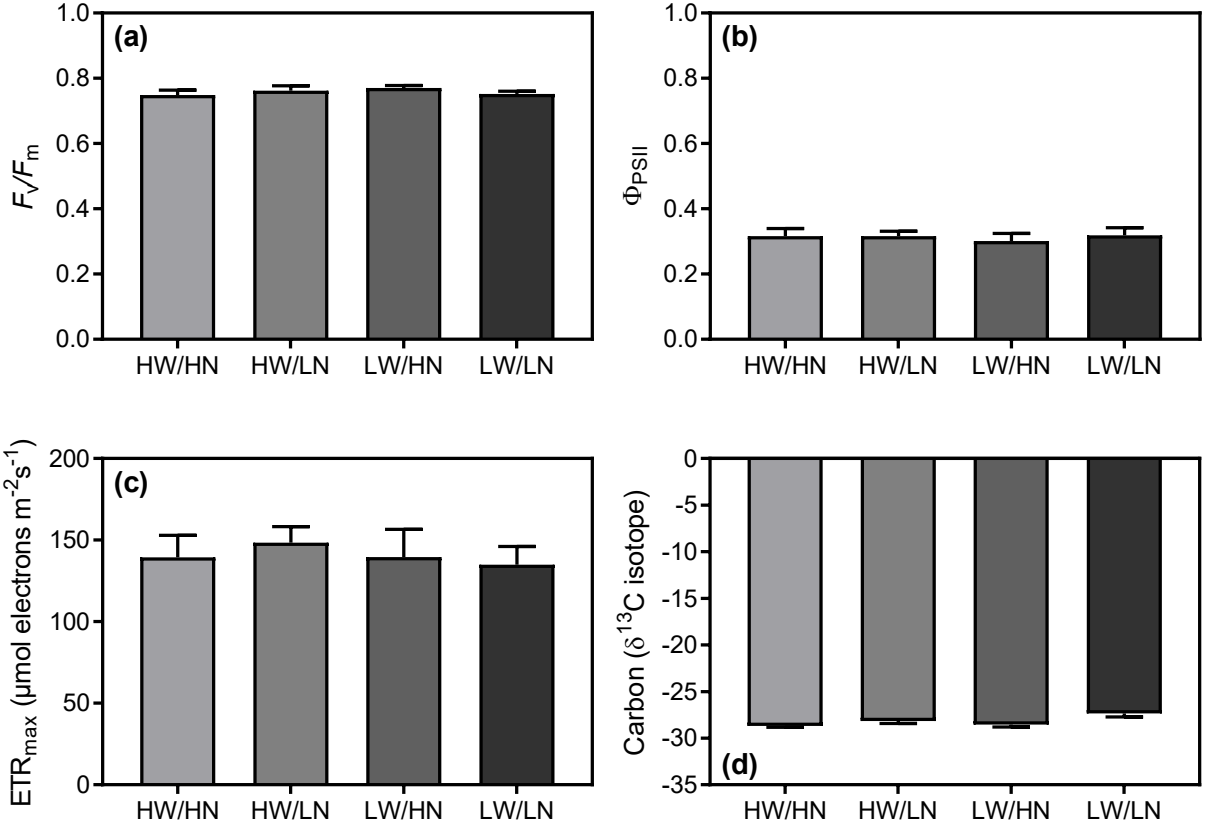
(b)
(c)
(d)
(e)
(f)
Total biomass (g DW)
Shoot biomass (g DW)
Root biomass (g DW)
125
100
75
50
25
0
80
60
40
20
30
10
a
b
c
HW/HN
HW/LN
LW/HN
LW/LN
Uninfected
Infected
HW
LW


(a)
Parasite biomass (g DW)
35
30
25
20
15
10
5
0
HW/HN
HW/LN
LW/HN
LW/LN
(b)
Parasite biomass (g host DW)
1.5
1.0
0.5
0.0
HW/HN
HW/LN
LW/HN
LW/LN


(a)
(b)
(c)
(d)
(e)
(f)
F_v/F_m
Φ_{PSII}
ETR_{max} (µmol electrons $m^{-2}s^{-1}$)
1.0
0.8
0.6
0.4
0.2
0.0
300
200
100
0
a
b
HW/HN
HW/LN
LW/HN
LW/LN
Uninfected
Infected


(a)
F_v/F_m
1.0
0.8
0.6
0.4
0.2
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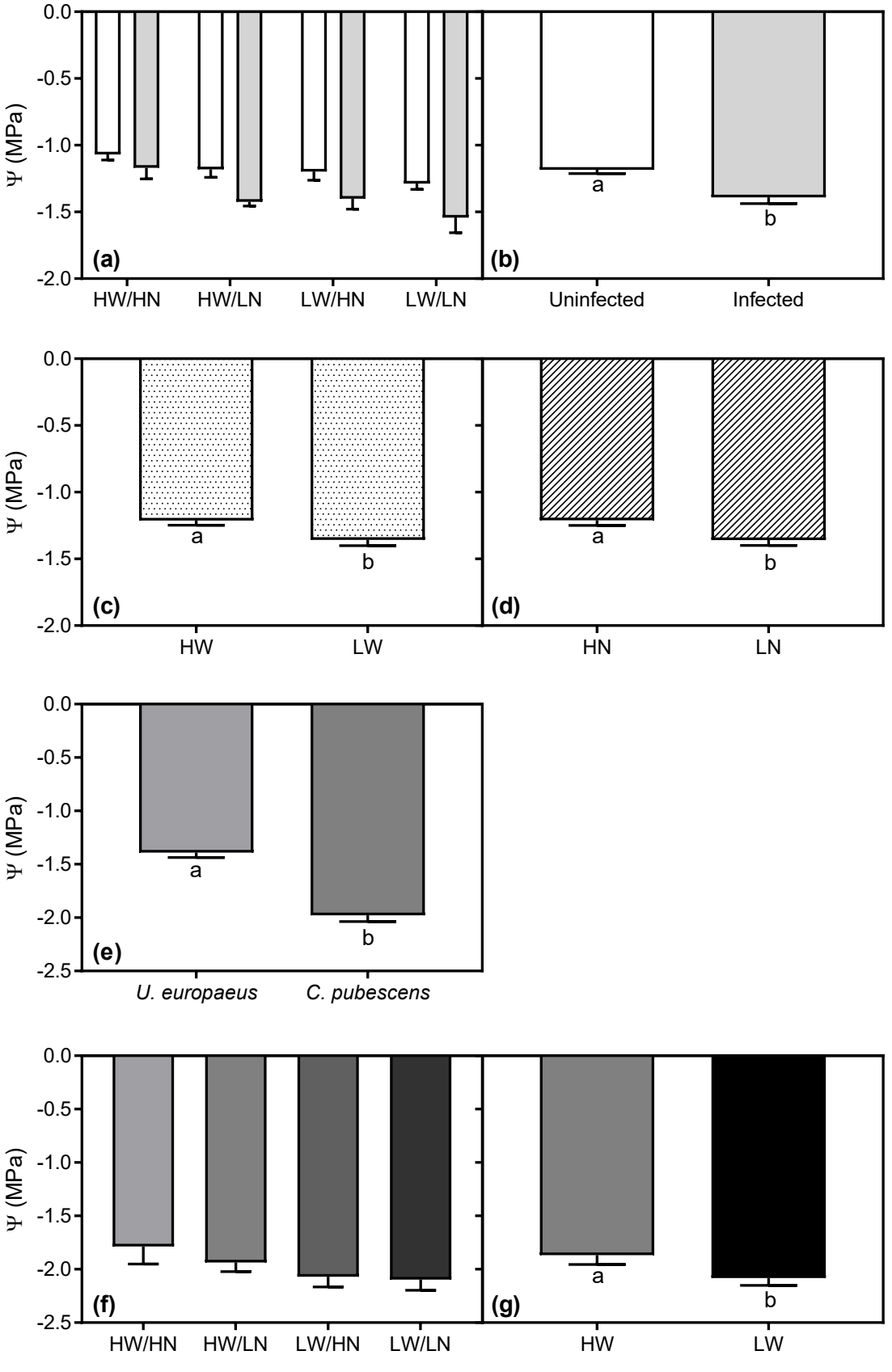
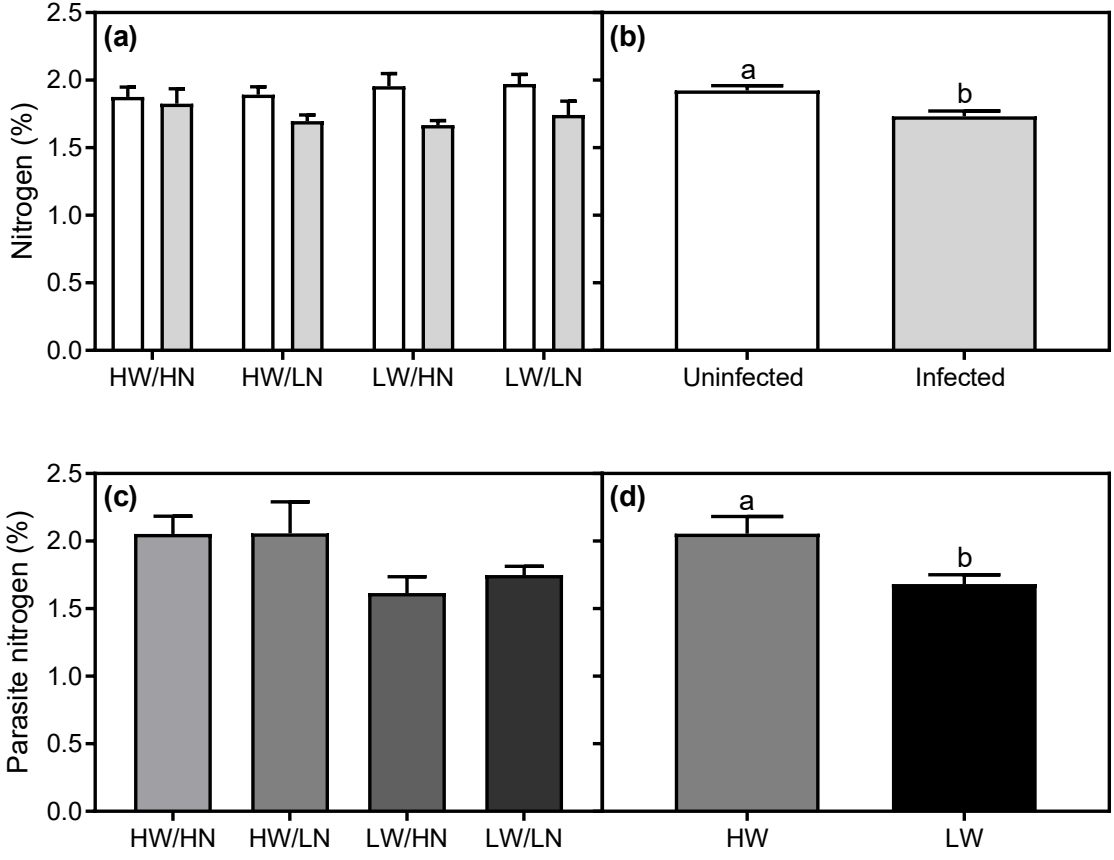
HW/HN
HW/LN
LW/HN
LW/LN
(b)
Φ_{PSII}
1.0
0.8
0.6
0.4
0.2
0.0
HW/HN
HW/LN
LW/HN
LW/LN
(c)
ETR_{max} (μmol electrons $m^{-2}s^{-1}$)
200
150
100
50
0
HW/HN
HW/LN
LW/HN
LW/LN
(d)
Carbon ($\delta^{13}C$ isotope)
0
-5
-10
-15
-20
-25
-30
-35
HW/HN
HW/LN
LW/HN
LW/LN


Ψ (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
(a)
HW/HN
HW/LN
LW/HN
LW/LN
(b)
a
b
Uninfected
Infected
(c)
HW
LW
(d)
HN
LN
(e)
U. europaeus
C. pubescens
(f)
(g)


(a)
(b)
(c)
(d)
Nitrogen (%)
Parasite nitrogen (%)
2.5
2.0
1.5
1.0
0.5
0.0
a
b
HW/HN
HW/LN
LW/HN
LW/LN
Uninfected
Infected
HW
LW